

Nystatin Production by a Local *Streptomyces* sp. Isolated from Egyptian Soil

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Abstract: Various soil samples were randomly collected at different places in south Delta, Egypt. A total of 105 *Streptomyces* isolates, were selected according to their morphological characteristics. Antifungal activity was assessed by agar well diffusion assay. Ten isolates showed antifungal activity against various reference strains of yeast and fungi. The maximum zone of inhibition was detected for *Aspergillus niger* and *Candida albicans* using cell homogenate of isolate N5. This potential isolate was identified on the bases of the International *Streptomyces* Project (ISP) as *Streptomyces noursei*. Accordingly the name *Streptomyces noursei* sp.N5 was proposed. Maximum antifungal productivity was observed on the 5th day of incubation at 30°C and at initial pH 7.0 in starch nitrate medium. The production medium was optimized to attain maximum productivity. Starch 1.0% (w/v), potassium nitrate 0.25% (w/v) and dipotassium hydrogen phosphate 0.1% (w/v) as carbon, nitrogen and phosphorus sources, respectively, were proved to give the highest significant antifungal productivity. Maximum antifungal production was detected at inoculum size of 2 % (v/v) in shake flasks containing 20 % (v/v) culture medium. The antifungal metabolite was efficiently extracted by methanol and was successfully recovered and purified by HPLC procedure. The spectral examination of the compound by Ultraviolet, Infrared, ¹H-NMR, ¹³C-NMR and Mass spectra (MS), suggested that it was Nystatin A3.

Key Words: *Streptomyces noursei*; optimization of conditions; nystatin; antifungal activity; agar well diffusion; productivity.

INTRODUCTION

The discovery and development of antibiotics was one of the most significant advances in medicine in the 20th century. Nevertheless, many antimicrobial agents that were used to treat a variety of human infectious diseases are now ineffective. Therefore, to ensure that effective drugs will be available in the future, it is necessary to improve the antimicrobial use patterns and to devise strategies to identify new antibiotics through previously unexplored targets^[27,20].

Microbial environment is an important source of novel active agents^[21]. Many of these products currently used are produced by microbial fermentation, or are derived from chemical modification of a microbial product^[9]. The fermentation process is an important tool for production of secondary metabolites that cannot be isolated from plants and animals, or synthesized by chemists^[7,29].

Streptomycetes have been, and remain, the most fruitful source of microorganisms for all types of bioactive metabolites. These secondary metabolites have important applications in human medicine (antiviral and anticancer compounds), in agriculture such as herbicides, insecticides and antiparasitic compounds^[31,33,1,8].

Invasive and disseminated fungal infections are serious clinical problems, especially among hospitalized and immunocompromised patients. The emergence of these opportunistic pathogens could be favored by the change in host susceptibility due to the growing number of immunocompromised individuals in the population as a result of HIV pandemic and the use of long-term immunosuppressive therapy in cancer and

organ transplant patients^[11]. Despite the development of newer antifungal drugs, the polyene antifungals including amphotericin B and nystatin, continue to be the most potent broad spectrum fungicides available for clinical use^[28,5]. As a consequence of the continuing search for new polyenes, as well as the limitations of current polyene production methods, research continues to be directed toward the improvement of polyene production and purification techniques^[12]. This study aimed at screening for antifungal activity from different local *Streptomyces* isolates from Egyptian soil, studying some of the physiological factors that could affect the antifungal productivity, purification and characterization of the produced antifungal metabolite.

MATERIALS AND METHODS

Microorganisms

Samples were randomly collected from soil at different places in south Delta, Egypt. *Streptomyces* colonies were selected according to their morphological characteristics. The tested microorganisms were obtained from MIRCEN Cairo, Faculty of Agriculture, Ein-Shams, University, Egypt. Long term preservation of isolates was achieved in soil stock cultures^[22].

Isolation of streptomycetes

One gram of soil sample was transferred to 10 ml sterile water. Isolation was carried out according to the dilution technique^[13]. Samples of the proper dilution were cultured by streak plate technique on starch nitrate, glycerol-asparagine and malt -yeast extract agar plates^[26,32] and incubated at 30.0±2°C

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for 1 - 2 weeks. The purified *Streptomyces* isolates were grown in starch nitrate broth medium consisting of (g/L): starch 10; KNO₃ 2.0; K₂HPO₄ 1.0; MgSO₄. 7 H₂O 0.5; NaCl 0.5; CaCO₃ 3.0; FeSO₄.7H₂O 0.01 adjusted to pH 7.0. Cell homogenate and broth supernatant of each isolate were screened, for their ability to produce antimicrobial metabolites in terms of their antimicrobial spectrum of activity determined by the agar diffusion method^[36], in comparison to control tetracycline and fluconazole discs containing (0.05mg/0.1ml). The most active isolate was chosen for further study.

Identification of *Streptomyces*

The active isolate was chosen, tested and identified on the bases of the observations made on International *Streptomyces* Project (ISP), recommended by Shirling and Gottlieb^[26].

Cultivation of the selected strain for antifungal metabolite production

One ml (2% v/v) of preincubated seed culture was transferred to 250 ml Erlenmeyer flasks containing 50 ml of starch nitrate medium adjusted at pH 7.0 using 0.1 N NaOH and 0.1 N HCl and was incubated on a rotary shaker (180 rpm) at 30°C for 5 days.

Medium optimization for maximum antifungal productivity

The effect of different carbon sources, nitrogen sources, phosphate salts, initial pH of culture medium ranging from pH 5.0 - 8.8 adjusted by 0.1 M citrate phosphate buffer (pH 5.0 -7.0), 0.1 M phosphate buffer (pH 7.0 &7.5) and 0.1 M Tris buffer (pH 8.0 & 8.8) and the effect of different incubation periods were carried out in shake flasks containing starch nitrate medium. Antifungal production was determined in terms of antifungal activity. *Aspergillus niger* was taken as a representative test organism to test the antifungal activity. All experiments were carried out in triplicate and results were expressed as the mean of inhibition zone \pm SD.

Extraction and purification of the antifungal metabolite

The microbial cells were collected at intervals, homogenized, bio-assayed by the agar diffusion method and freeze- dried. The freeze- dried mycelia were extracted with methyl alcohol at pH value \approx 7.0. The extracts were combined, filtered then concentrated under vacuum to \approx 20.0% of its volume and the active substance was precipitated with acetone. TLC was performed by spotting the active substance dissolved in methanol on a pre-coated plates of silica gel using methanol:chloroform (9.0 : 1.0 v/v and 8.0 : 2.0 v/v) as mobile phase in an ascending manner. The resulting spots were detected at UV light and sprayed with vanillin sulfate to detect the bands. The sample was subjected to separation by HPLC procedure as a final purification.

Structural elucidation of the purified antifungal metabolite

The elemental analysis for the presence of carbon, hydrogen, nitrogen, and oxygen in the antifungal metabolite was estimated. The purified active compound obtained was subjected to various examinations by: Ultraviolet, Infrared, ¹H-NMR, ¹³C-NMR and Mass spectra (MS). Those were done at the Micro-analysis Center, Cairo University and National Research Center, Cairo-Egypt.

Statistical Methodology

Statistical analysis was done through SPSS software, version 16.0. Comparisons between the results of the different compounds were done using Analysis of Variance (ANOVA) and the Post-hoc test least significant differences (LSD). The significance level was considered at P-value < 0.05.

RESULTS AND DISCUSSION

Exploration of novel sources of antimicrobial drugs remains a continuous challenge in pharmaceutical biotechnology. Several investigations have been done for screening new isolates of actinomycetes or producing new antimicrobial agents. ^[2,8, 18,19; 30]

Table 1. Antibiosis of *Streptomyces noursei* sp. N5 towards various reference strains.

Test organisms	Mean Inhibition zone diameter (mm) Isolate N5	Mean Inhibition zone diameter (mm) Tetracycline	Mean Inhibition zone diameter (mm) Fluconazole
Bacteria:			
<i>Bacillus cereus</i> NRRL B-569	15		
<i>Bacillus subtilis</i> NRRL 4219	13		
<i>Staphylococcus aureus</i> ATCC 29213	14	20	
<i>Escherichia coli</i> ATCC 25922	11	16	
<i>Pseudomonas aeruginosa</i> ATCC 27953	11		
Fungi:			
<i>Aspergillus flavus</i> NRRL 3357	29		
<i>Aspergillus niger</i> NRRL 328	35		13
<i>Aspergillus terreus</i> NRRL 255	30		
<i>Botrytis allii</i> NRRL 2502	32		
<i>Diplodia oryzae</i> NRRL 1336	28		
<i>Fusarium oxysporium</i> NRRL 3307	31		
<i>Helmentosporium turcicum</i> NRRL 4671	27		
<i>Machrophomina phaseoli</i> ATCC 20441	28		
<i>Trichoderma viride</i> NRRL 8044	31		
Yeast:			
<i>Candida albicans</i> ATCC 10231	25		15
<i>Candida pseudotropicalis</i> ATCC 4135	22		
<i>Rhodotorula minuta</i> ATCC 10658	18		

Ten isolates out of 105 *Streptomyces* isolates showed antifungal activity against various strains of yeast and fungi. Antifungal activities were detected either in cell homogenate and supernatant (Data not shown). Maximum antifungal activity was detected in the cell homogenate of isolate N5, especially against *Aspergillus niger* and *Candida albicans* compared to fluconazole, while weak activities were detected against bacteria (Table. 1). The isolate with the highest activity (isolate N5) was considered for identification. The microscopic examination of isolate N5 revealed that the spore chain morphology was spiral (Figure. 1a) and the spore surface was spiny (Figure. 1b). The cultural characteristics showed that, the isolate N5 grew well on the tested media either organic or synthetic media (starch nitrate, glycerol asparagine, yeast-malt extract, oat meal, fish-meal extract, soya- bean meal). The aerial mass color was in the gray-color series. The substrate mycelium colors varied depending on the type of the media used. The obtained colors of the examined strains were matched with those of charts colors [14]. The colors both aerial and substrate mycelia of the isolated strain was similar or closely related to certain reference species and in agreement with those described in the ISP. The diffused pigments were sometimes observed (data not shown).The physiological and biochemical characteristics of isolate N5 are listed in Table (2). Melanoid pigments are not formed in peptone-yeast extract-iron agar or tyrosine agar. Weak proteolysis, lipolysis, and lecithinase activities and no chitin or pectin decomposition were detected by this isolate. The utilization of various carbohydrates by the selected isolate N5 revealed that most of the tested sugars were well utilized. Weak growth was noticed with sucrose and raffinose and no growth on L-arabinose, D-xylose, rhamnose and also, on the agar control medium without carbon source. This isolate tolerated NaCl up to 6% indicating that it is highly halophilic. Growth was recorded at a temperature range of 20 to 35°C and at pH range of 6.0 to 8.0. According to the determinative keys, the taxonomical identification of isolate N5 showed that it belonged to the genus *Streptomyces*. It was likely to be *Streptomyces noursei* and was designated as *Streptomyces noursei* sp.N5.



Figure 1a. Photograph of spore chains of *Streptomyces noursei* sp. N5 (X3000).

Table 2. Physiological and biochemical characteristics of *Streptomyces noursei* sp. N5.

Character state	Results
Melanin pigment production:	
- Peptone-yeast extract-iron agar	-
- Tyrosine agar	-
Enzyme activities:	
- Proteolysis	±
- Lipolysis	±
- Lecithinase	±
Hydrolysis activities:	
- Chitin	-
- Pectin	-
- Gelatin	±
Nitrate reduction:	+
H2S Production:	±
Utilization of different carbon sources:	
- Control: Negative control	-
Positive control (Glucose)	+
- L-arabinose	-
- D-xylose	-
- I-Inositol	+
- D-mannitol	+
- D-fructose	+
- Rhamnose	-
- Sucrose	±
- Raffinose	±
- D- galactose	+
Tolerance to NaCl:	6%
Growth temperature:	20-35°C
pH range of growth:	6.0-8.0

Negative control: No sugars **Positive control :** Glucose - : Negative result + : Positive result ± : Doubtful (weak, variable or trace of growth).



Figure 1b. Electron micrograph of spore surface of *Streptomyces noursei* sp. N5 (X16000).

The microbial production of secondary metabolites is extremely sensitive to environmental factors or culture conditions [3, 17]. For instance, the in vitro production of most antibiotics depends on the composition of the culture medium in which the producer organism is grown. For this reason, medium optimization has been the standard procedure for optimizing antibiotic production [20]

A study on the production of antifungal metabolite by *S.noursei* sp.N5, may be considered as a prelude for the optimization of some cultural conditions to attain maximum antifungal activity. Starch nitrate culture medium was used as a basal medium for fermentation in shake flasks to investigate the effect of various carbon, nitrogen, phosphate salts, inoculum sizes, aeration,

agitation, initial pH values and incubation period on the antifungal metabolite productivity.

Among the tested carbon sources, starch supported the highest antifungal metabolite production by *S. noursei* sp. N5, followed by lactose, galactose, sucrose, maltose, mannose, glucose, fructose and mannitol respectively, while the least productivity was obtained when the medium was supplemented with xylose, raffinose, arabinose and rhamnose (figure.2). This may be explained by the inability of these sugars to support growth as indicated by the pattern of different carbohydrate utilization by this strain. Although sucrose weakly supported growth, it exhibited reasonable productivity, however, such growth limitation might trigger the onset of secondary metabolite production.

There was no significant difference between starch concentrations of 10, 12.5, and 15 (g/L) (Data not shown). Many authors studied the effects of different carbon sources and initial sugar concentration on yield of cells and metabolites [23]. In a study of Ripa et al. [24] the authors demonstrated that, glucose 2% was the best carbon source for antimicrobial metabolite production by *Streptomyces* sp. RUPA-08PR. In contrast, Demain and Fang [6] reported that carbohydrates such as glycerol, maltose, lactose and some others are known to have interference with the production of secondary metabolites. In that context, Jonsbu et al. [15], reported that, in case of *Streptomyces* species, with regards to carbon sources species specific variation may occur for cell growth and secondary metabolites production.

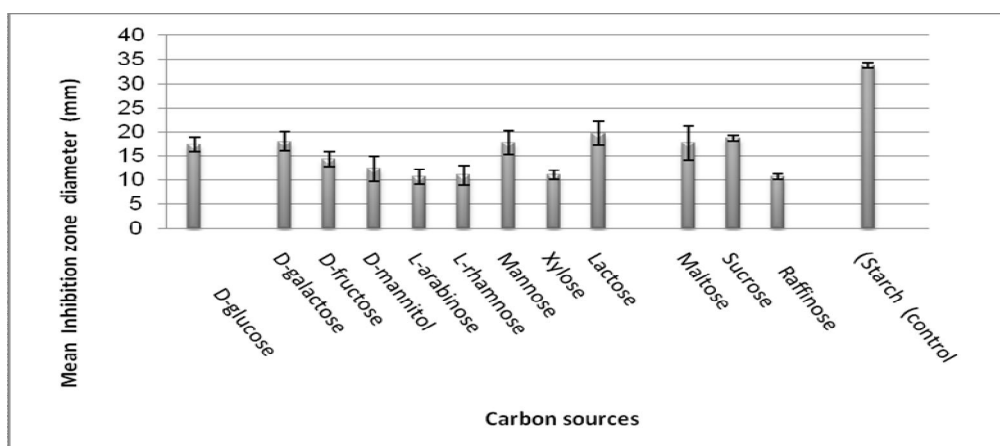


Figure 2. Effect of different carbon sources on antifungal metabolite productivity by *Streptomyces noursei* sp. N5. Data are average of three replicate culture flasks, error bars show s.d.

Ammonium salts and nitrate salts, especially potassium nitrate elicited significantly higher antifungal metabolite production than other tested inorganic or organic sources (Figure 3). The optimum concentration was 2.5 g/L, though there was no significant difference between concentrations of 2.0, 2.5 and 3.0 (g/L) (Data not shown). In a study of Jonsbu et al. [14] the authors

found that *Streptomyces noursei* was able to utilize ammonium nitrate, sodium nitrate and ammonium sulphate for the growth and production of nystatin, high ammonium concentration had a negative effect on production of nystatin when phosphate and glucose was in excess, and that there was an increased production of nystatin when the cultures became ammonium limited.

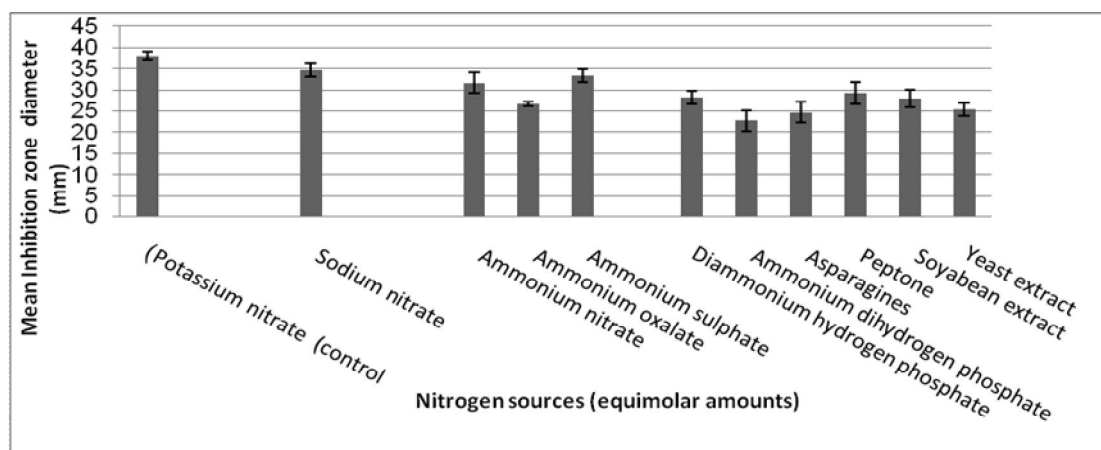


Figure 3. Effect of different nitrogen sources on antifungal metabolite productivity by *Streptomyces noursei* sp. N5. Data are average of three replicate culture flasks, error bars show s.d.

The level of antifungal metabolite production produced by *Streptomyces noursei* sp.N5 was optimum with dipotassium hydrogen phosphate salt and was significantly higher than the other tested phosphate salts (figure. 4). This result was in agreement with Ripa et al^[24], who found that K₂HPO₄ had positive effects on antibiotic production by *Streptomyces* sp. RUPA-08PR. Thus, in general the basic phosphates were more preferable for antifungal metabolite production. This was explained by De Lorenzo (1985)^[4] and Raytapadar & Paul (2001)^[23], who reported that, the

absence of mineral sources of phosphorous in the medium causes a substantial drop in the activity and a decrease in the intensity of growth of the culture, which is due not only to the significance of phosphorous as an element of nutrition, but also to the buffering of the solutions of its salts. Optimum concentration of dipotassium hydrogen phosphate was 1.5 g/L compared to the tested concentration range of 0.25- 2.5 g/L, however no significant difference was detected between 1 and 1.5 g/L (Data not shown)

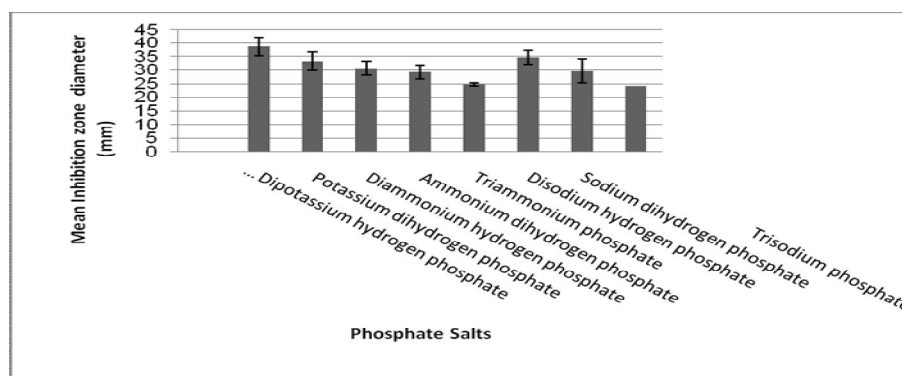


Figure 4. Effect of different phosphate salts on the antifungal metabolite productivity by *Streptomyces noursei* sp. N5. Data are average of three replicate culture flasks, error bars show s.d.

Regarding the fermentation conditions, maximum productivity was detected using inoculum sizes 2 and 3% (v/v), a noticeable repression on the antifungal metabolite production was detected at lower & higher inoculum sizes while there was no significant difference between 2 and 3% (Data not shown). Productivity was also affected by the initial pH values of the culture medium and was observed over pH range of 6-8. This finding might be justified by the effect of the culture pH, during fermentation, on many microbiological processes and the transport of various components across the cell membrane^[10]. The optimum antifungal metabolite production was observed using culture medium adjusted at pH 7.0. with 0.1 N HCl and

0.1 N NaOH. At extreme pH values no productivity was detected (Figure. 5). It was also found that, fermentation in shake flasks containing 20 % (v/v) culture medium had a positive influence on production compared to 30 and 40% (v/v). No significant difference in productivity was observed between agitation at 150,180 and 200 rpm (Data not shown). However, there was a markedly significant decrease in antifungal metabolite production at 100 and 120 rpm. Thus, the increase in sample volume, and decreased agitation rate might reduce the dissolved oxygen level in fermentation broth that could affect the antifungal metabolite production.

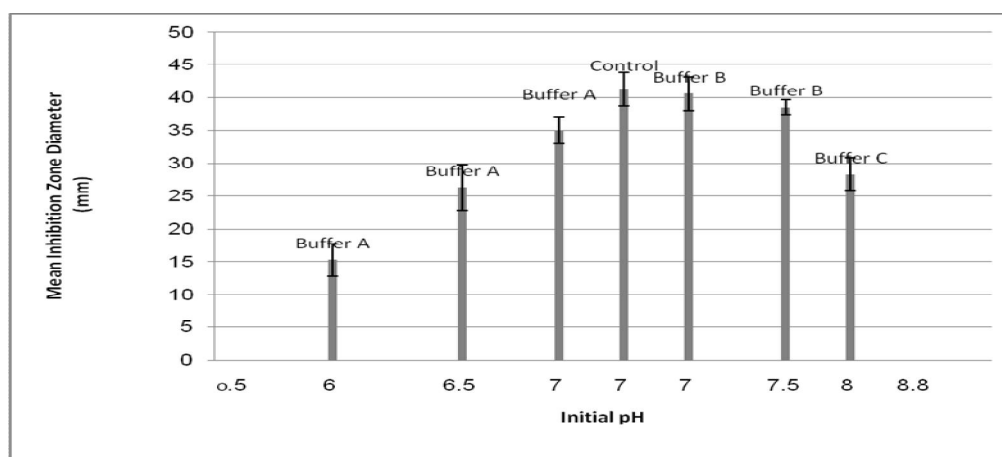


Figure 5. Effect of initial pH values on the antifungal metabolite productivity by *Streptomyces noursei* sp. N5. Data are average of three replicate culture flasks, error bars show s.d. Buffer (A) : Citrate phosphate buffer solution , Buffer (B) : Phosphate buffer solution , Buffer (C) : Tris buffer solution. Control : Optimized basal medium adjusted with 0.1 N NaOH or 0.1 N HCl to pH 7.0.

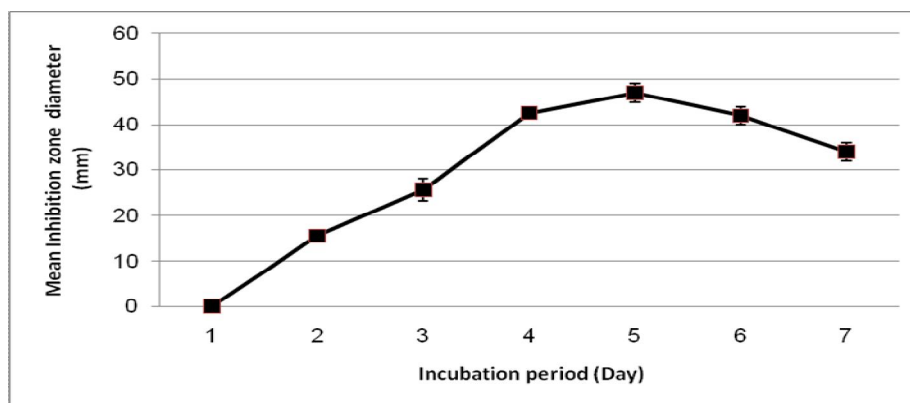


Figure 6. Time course of the antifungal metabolite productivity *Streptomyces noursei* sp. N5. Data are average of three replicate culture flasks, error bars show s.d.

During fermentation of *Streptomyces noursei* sp. N5, in optimized media, production of antifungal metabolite started after a lag period of 24 h, then a gradual increase in productivity was observed till reaching a peak at the 5th day, thereafter there was a gradual decline (Figure. 6). This trend of productivity is representative for the typical pattern of streptomycetal growth, in which productivity reached a peak during the stationary phase.

The antifungal metabolite produced by *Streptomyces noursei* sp. N5 was efficiently extracted by methanol. Alcohols are the solvents most commonly employed in industrial polyene extraction [35]. Despite the careful, methodical refinement of the broth and mycelial

nystatin extraction protocols, nystatin recovery techniques still tend to produce relatively impure, low potency products that require further purification [8]. In the present work, the antifungal metabolite extract was subjected to further testing and purification by TLC and HPLC. Two spots were detected by TLC and their activities were tested against *Aspergillus niger* using paper disc assay method. Only, one spot showed considerable activity. The sample was subjected to separation by HPLC. Two compounds were eluted, showing two peaks, one active peak that was closely related to the active peak of nystatin as a standard sample. The other peak (the smallest one) showed no antimicrobial activity (Figure. 7).

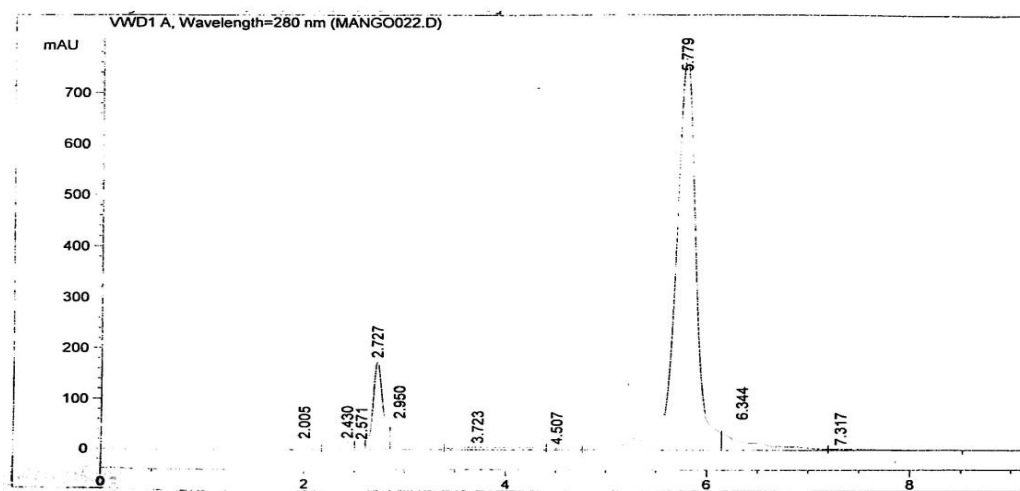


Figure 7. HPLC chart of the antifungal metabolite produced by *Streptomyces noursei* sp. N5

The antifungal metabolite produced by *Streptomyces noursei* sp. N5 gave positive Molish test indicating its glycosidic nature. It was investigated by different spectral methods via MS, IR, UV, HNMR and C^{13} NMR. UV absorption was at nm 285, 306 and 330. Also, the IR spectrum of antifungal metabolite gave characteristic bands of cm^{-1} 3744 (COOH), 3420 (NH and OH), 2926 (CH, CH_2 , CH_3), 1743 and 1648 (CO) and 1518 (C=C). The HNMR and C^{13} NMR spectrum of antifungal agent data were compared with the published data, which supported that the deduced structure was Nystatin A3. Nystatin A3 has a molecular formula

$C_{53}H_{85}NO_{20}$ for molecular weight of 1055, however the mass spectrum (MS) of the antifungal agent gives M^+ at m/z 902 and this could be explained as shown in figure (8) by rearrangement of the molecular ion and opening the cyclic terminal two sugars to the straight chain form and loss of $C_3H_5O_2$ in ionic form to give base peak at m/z 73, this is followed by loss of fragments, H_2O , OH, NH_2 , CH_3 and CH_2 from the other terminal sugar to give M^+ AT M/z 902. The mass spectrum (Figure 9.) showed different loss of CH, CH_2 , OH, CO, CO_2 supporting the nature of this compound.

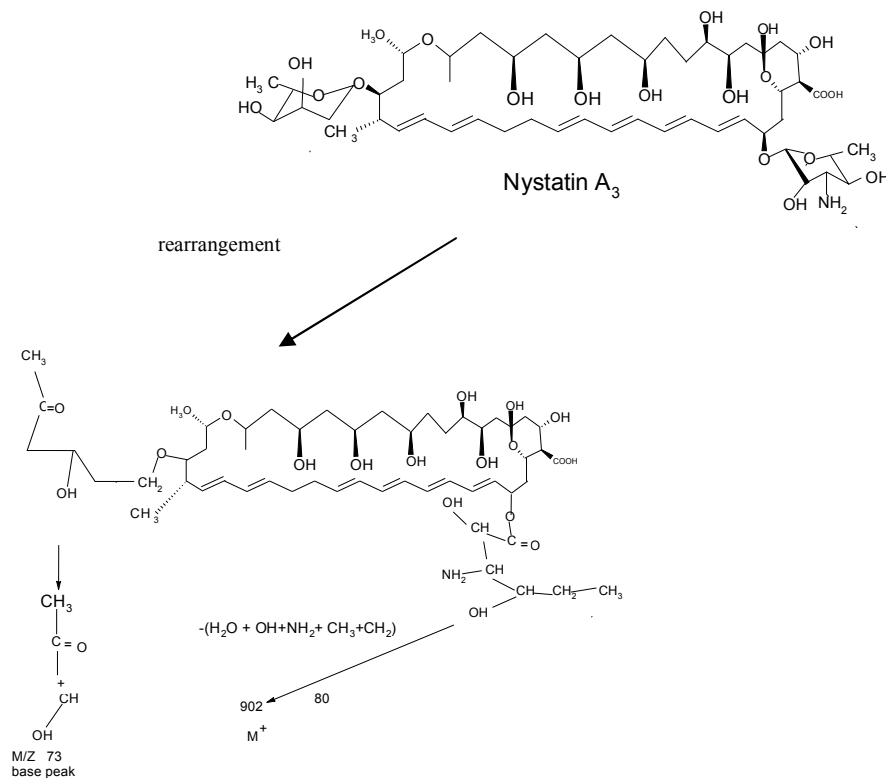


Figure 8. Deduced structure of the purified antifungal metabolite produced by *Streptomyces noursei* sp. N5

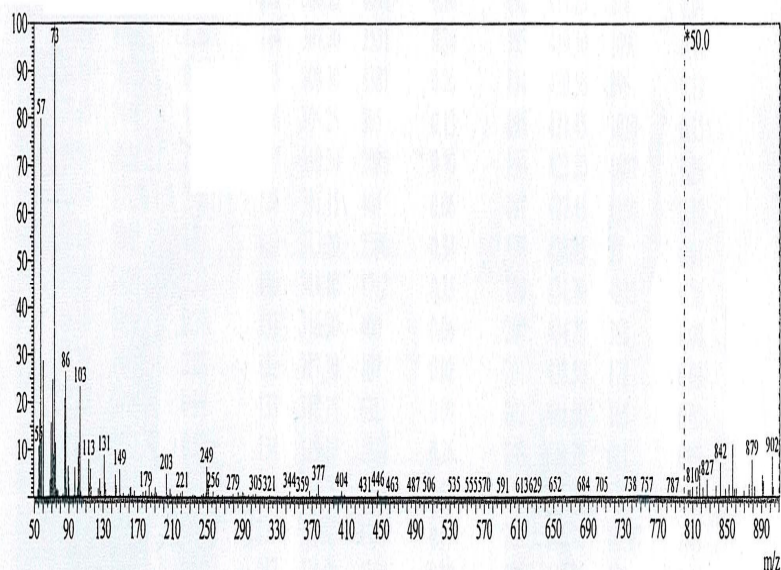


Figure 9. Mass spectrum of the purified antifungal metabolite produced by *Streptomyces noursei* sp. N5

CONCLUSION

Streptomyces noursei sp. N5 is a promising candidate for nystatin production under optimized conditions. Further studies should be done for large scale production of nystatin by our local isolate.

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